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Transfer and mapping of the heat tolerance component traits of *Aegilops speltoides* in tetraploid wheat *Triticum durum*

Zewdu Teshome Awlachew · Rohtas Singh · Satinder Kaur · Navtej S. Bains · Parveen Chhuneja

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Abstract *Aegilops speltoides* is an important genetic resource for wheat improvement and has high levels of heat tolerance. A heat-tolerant accession of *Ae. speltoides* pau3809 was crossed with *Triticum durum* cv. PDW274, and $BC_2F_{4\text{-}6}$ backcross introgression lines (BILs) were developed, phenotyped for important physiological traits, genotyped using SSR markers and used for mapping the QTL governing heat tolerance component traits. A set of 90 BILs was selected from preliminary evaluation of a broader set of 262 BILs under heat stress. Phenotyping was conducted for physiological traits such as cell membrane thermostability, chlorophyll content, acquired thermotolerance, canopy temperature and stay green. Much variation for these traits was observed in random as well as selected sets of BILs, and comparison of the BILs with the recurrent parent showed improvement for these traits under normal as well as heat stress conditions, indicating that introgressions from *Ae. speltoides* might have led to the improvement in the heat tolerance potential of the BILs. Introgression profiling of the 90 BILs using SSR markers identified *Ae. speltoides* introgression on all the 14 chromosomes with introgressions observed on A as well as B genome chromosomes. QTL mapping identified loci for various heat tolerance component traits on chromosomes 2B, 3A, 3B, 5A, 5B and 7A at significant LOD scores and with phenotypic contributions varying from 11.1 to 28.7 % for different traits. The heat-tolerant BILs and QTL reported in the present study form a potential resource that can be used for wheat germplasm enhancement for heat stress tolerance.

Keywords *Aegilops speltoides* · Canopy temperature · Cell membrane thermostability · Chlorophyll content · Stay green · Heat tolerance · QTL mapping · SSR markers

Abbreviations

BIL	Backcross introgression line
CC	Chlorophyll content
CMS	Cell membrane thermostability
CT	Canopy temperature
HSE	Heat stress environment
OE	Optimum environment
SPAD	Soil plant analysis development
ATT	Acquired thermo tolerance

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Z. T. Awlachew · R. Singh · S. Kaur · P. Chhuneja (✉)
School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana 141004, India
e-mail: pchhuneja@pau.edu

N. S. Bains
Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana 141004, India

Present Address:
Z. T. Awlachew
Department of Biology, University of Gondar, Gondar, Ethiopia

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QTL Quantitative trait loci
SG Stay green
Vp Vegetative period

Introduction

Wheat occupies the second position after rice among major cereals as a main source of the world's food energy and nutrition. The major wheat-producing countries are China, India, the USA, the Russian Federation and Australia in addition to Canada and the European Union. These countries together contribute more than half of the global wheat production. India contributed about 90.78 million tons from 30.37 million hectares in 2015 (http://agricoop.nic.in). In India, wheat cultivation has traditionally been dominated by the northern region, and the plains of the Punjab and Haryana states are prolific wheat producers. Heat stress is the major abiotic constraint to wheat productivity and specifically the terminal heat stress, which has been found to affect the critical reproductive growth stages, mainly anthesis and grain filling (Wahid et al. 2007), in most of the wheat-growing regions of the world, including India. In India alone, as much as 13.5 million ha of the wheat-growing area is very often subjected to heat stress (Joshi et al. 2007). A significant portion of wheat grown in south Asia is also affected by heat stress.

A rise in temperature at the time of grain filling, referred as "terminal heat stress," is responsible for the decline in wheat production in many environments around the world, covering 36 million ha (Reynolds et al. 2001; Hays et al. 2007). It has been estimated that a 1 °C rise in temperature beyond 18 and 22 °C during grain filling in wheat reduces the grain filling period by 5 % and reduces the grain yield by 3–4 % (Gupta et al. 2012). Using this parameter, it has been projected that most commercially grown wheat cultivars in India would suffer a loss of up to ~50 % in their yield potential when exposed to 32–38 °C at the crucial grain formation stage. As the temperature rises above 18–22 °C, the observed decrease in the duration of deposition of dry matter in the kernel is not accompanied by a compensating increase in the rate of grain filling with the result that the grain weight and yield are diminished at high temperatures (Jenner 1994).

Heat stress is a complicated trait, and selection for yield under heat stress is difficult in the segregating generations in the breeding programs. The increased membrane thermostability, stay green traits, canopy temperature and other physiological parameters might be helpful for screening the heat-tolerant genotypes that have been used in various studies for assessing the thermotolerance potential of wheat genotypes (Cossani and Reynolds 2012; Prerna et al. 2013). High temperature reduces the chlorophyll and the photosynthetic capacity of leaves (Prasad et al. 2008). Thylakoid membranes of the chloroplasts are one of the most heat-sensitive cellular components, and damage to the thylakoid membranes results in chlorophyll loss, compromising photosynthetic efficiency (Pradhan et al. 2012). High temperature stress leads to increases in the leaf temperature, thereby increasing canopy temperature, which is an indirect measure of (instantaneous) transpiration at the whole-crop level (Reynolds 2002) and plant water status (Araus 2003). Wheat genotypes with a low canopy temperature can maintain high transpiration and photosynthetic rates as well as produce a high yield (Talebi 2011; Calderini et al. 1999). Chlorophyll content and CT have been used for selection for heat stress tolerance in wheat (Balota et al. 2007).

The most promising approach to alleviating the effect of heat stress on yield is to develop stress-tolerant varieties (Wahid et al. 2007). *Aegilops* species have been considered a genetic resource for increasing the genetic potential of cultivated wheat to withstand biotic and abiotic stresses (Pradhan et al. 2012). In various studies, *Ae. speltoides*, *Ae. tauschii* and *Ae. geniculata* have been identified to be highly thermotolerant (Ehdaie and Waines 1992; Waines 1994; Khanna-Chopra and Viswanathan 1999; Zaharieva et al. 2001; Gupta et al. 2010; Pradhan et al. 2012). The evaluation of wild *Triticum* and *Aegilops* species at our institute also led to the identification of *Ae. speltoides* as highly tolerant to heat stress. In the present study, development of *Triticum durum-Ae. speltoides* backcross introgression lines, physiological evaluation of backcross introgression lines under normal and heat stress conditions and QTL mapping for heat tolerance component traits is reported.

Materials and methods

Plant material

Visual screening of wild *Triticum* and *Aegilops* species showed that *Ae. speltoides* has a high level

of heat tolerance, which was indicated by the stay green trait, normal pollen fertility and seed setting at temperatures as high as 39 °C (data not given). To transfer heat tolerance to cultivated wheat, a thermotolerant accession of *Ae. speltoides* ($2n = 2X = 14$) pau3809 was crossed as the pollen parent with tetraploid *T. durum* cv. PDW274 ($2n = 4X = 28$) as the female parent. The ten F_1 seeds were obtained and F_1 plants tillered profusely, and they were extensively backcrossed with PDW274. More than 1600 florets were pollinated, and only 5 BC_1F_1 plants were obtained, which were again extensively backcrossed; 128 BC_2F_1 plants were obtained, which were selfed to generate BC_2F_2 progenies. The crossing strategy used for the development of the material is depicted in online Figure S1. All the BC_2F_2 plants were then carried forward, and homozygous backcross introgression lines were developed. The meiotic analysis in F_1, BC_1F_1 and BC_2F_1 was conducted by fixing spikes at the pre-booting stage in Cornoy's fixative (6 ethanol:3 chloroform:1 glacial acetic acid) and preparing chromosome spreads from pollen mother cells (PMCs) through the squash method in 2 % acetocarmine solution.

Raising of the BILs

A random set of 262 BC_2F_4 introgression lines (BILs) was planted in the 2010–2011 cropping season in an augmented design along with six checks at two sowing dates, optimal (12 November 2010), which exposed BILs to the optimum growing conditions encountered by the wheat in the North Western Plains Zone, and late sowing (16 December 2010), which simulated terminal heat stress conditions. In the second year (2011–2012), on the basis of the 2010–2011 data analysis, 90 BILs were selected, which were planted in a factorial randomized block design with three replications again under optimal (4 November 2011) and late sown conditions (20 December 2011). Each genotype was grown in 1.5-m pair rows with row-to-row spacing of about 20 cm. All standard recommended agronomic practices, i.e., hoeing, weeding, irrigation, etc., were adopted uniformly, and data were recorded at an appropriate time for each trait. Normal sown and late sown conditions will be referred to as the optimum environment (OE) and heat stress environment (HSE) from hereon in the article.

Physiological trait measurement

Chlorophyll content

The total chlorophyll content was measured in intact leaves using a portable chlorophyll meter [SPAD-502, Soil Plant Analysis Development (SPAD) Section, Minolta Camera Co, Osaka, Japan] at vegetative (CCV) and anthesis stage (CCA) for both BILs grown under OE and HSE during 2010–2011 and 2011–2012. For this, five readings were taken at the vegetative and anthesis stage along the middle section of the leaf, and means used for analysis and values were expressed as SPAD units. Data were collected from 11 a.m. to 3 p.m. in full sun and calm weather conditions.

Cell membrane thermostability

The assay was performed according to Sadalla et al. (1990) with some minor modifications. It was measured for 262 BILs at two stages, vegetative and anthesis (CMTV and CMTA), in both the OE and HSE set during 2010–2011 only. Six leaf samples (7 cm long) from each BIL were washed three times with deionized water and taken in a capped vial. Sample vials were then kept for 1 h in a water bath preheated to 49 °C for administering heat shock. After heat treatment three replications from each BIL, i.e., two leaves per vial, were prepared, and 15 ml of deionized water was added to each vial. All vials were placed for incubation at 10 °C for 24 h followed by room temperature for 1 h, and the conductivity was recorded ($T1$) using a digital conductivity meter (Model CON 510, Eutech Instruments, India). After measuring the conductivity, the vials were autoclaved for 15 min at 121 °C, and their conductance ($T2$) was measured again. Membrane thermostability was calculated in percentage units as the reciprocal of relative leakage according to Ibrahim and Quick (2001) as follows:

$$\text{CMT}\,(\%) = [1 - (T1/T2)] \times 100$$

Cell viability assay

TTC (2,3,5-triphenyltetrazolium chloride)-based cell viability is another method that measures CMT and is used for estimation of tolerance to heat stress. This assay was done according to Ibrahim and Quick

(2001) with minor modifications at the anthesis stage in both OE and HSE sets of 90 selected BILs in the 2011–2012 season. Two leaf samples in triplicates in two sets (3.5 cm long) were collected from each BIL, washed thrice with double-distilled water, excised in small pieces and placed in a test tube containing 100 µl deionized water. The first set was left at 25 °C for 1 h for use as control while a second set of samples kept at 49 °C for 1 h in a preheated water bath was used as heat stressed samples. After this, in both the treatments, 8 ml solution of 0.8 % TTC (w/v) (2, 3, 5-triphenyltetrazolium chloride dissolved in a 0.05 M $NaPO_4$ buffer, pH 7.4) was added to each tube, and the tubes were placed in a vacuum chamber for 10 min to infiltrate TTC into leaf tissues. Leaf tissues were considered to be infiltrated when they settled at the bottom of the tube after release of the vacuum. Then the tubes were capped and incubated in total darkness for 24 h at 25 °C. After incubation, the TTC solution was drained off, and the leaf samples were washed three times with double-distilled water. Formazan was extracted by adding 3 ml of 95 % ethanol at 25 °C for 24 h in darkness. The amount of formazan dye produced by TTC reduction was measured with a spectrophotometer by reading the optical density (OD) at 530 nm. Cell viability as a measurement of thermotolerance was determined as the per cent of treated leaves relative to the control absorbency and expressed as acquired thermotolerance (ATT) as:

$$\text{Acquired thermotolerance}\,(\%) = (\text{OD}_\text{h}/\text{OD}_\text{c}) \times 100$$

where OD_h represents OD for the heat-stressed sample and OD_c for the control sample.

Canopy temperature

Canopy temperature (CT) was measured by a hand-held infrared thermometer (Model 8866, JQA Instrument, Inc., Tokyo, Japan) according to Bahar et al. (2008) at ZGS 6.9 (Zadoks Growth Scale) at the completion of anthesis (Zadoks et al. 1974) in 90 BILs during the 2011–2012 season. Data were taken in triplicates in the late morning to early afternoon during cloudless days and from the same side of each plot at an angle of approximately 45° to the horizontal in a range of directions such that they covered different regions of the plot and integrated many leaves. This procedure was followed to minimize the influence of exposed soil.

Stay green habit

Stay green habit (SG) was evaluated based on visual scoring (1–5 scale) of the greenness of leaves and spike for 90 BILs during the 2011–2012 season. Scores were given as (1) when both the leaves and spike were completely dry; (2) the leaves were dry and ear partially green; (3) both the leaves and ear were partially green; (4) leaves were partially green and the ear completely green; (5) both the leaves and ear were totally green. Scoring was done five times in intervals of every 3 days.

Heat susceptibility index

The heat susceptibility index (HSI) for heat tolerance component traits was calculated using the formula of Fischer and Maurer (1978)

$$\text{HSI} = \left(1 - Y/Y_\text{p}\right)/D$$

where Y is the average trait value of a BIL under heat stress (HSE), Y_p is the average trait value of the same BIL under optimum growth conditions (OE), and D is the stress intensity equal to $1 - X/X_\text{p}$, in which X is the mean Y of all BILs and X_p is the mean Y_p of all BILs. Accessions were classified as highly tolerant ($\text{HSI} \leq 0.5$), moderately tolerant ($0.5 < \text{HIS} \leq 1.0$) or susceptible ($\text{HSI} > 1.0$) to HT stress (Viswanathan and Khanna-Chopra 2001).

Statistical analysis

The data recorded for all the physiological traits were subjected to analysis of variance (ANOVA) with SAS (SAS Institute, USA) for an augmented design, and mean differences were compared using LSD at a 5 % probability level. For analysis of the selected set of 90 BILs ANOVA for RBD was run using the CropStat statistical program, a computer software, version 7.2.3 (International Rice Research Institute). Student's paired t test was applied using SigmaPlot 11.0 on the mean values of all the genotypes grown under two conditions, OE and HSE, and their level of significance was determined. Box plots were also made using SigmaPlot 11.0.

Meteorological data

Metrological data were procured from the School of Climate Change and Agricultural Meteorology of our institute to determine the differences in temperature to which optimal and late sown BILs were exposed. Means of maximum and minimum temperature of 10 weeks in the month of March and April, the most crucial phase for terminal heat stress in wheat, were used.

QTL mapping

Simple sequence repeat (SSR) analysis

Fresh leaves were collected from the 90 BILs and grounded in liquid nitrogen. DNA was extracted from the grounded leaf tissue using the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1990) with minor modification. A and B genome-specific SSR primers (Somers et al. 2004) were used for molecular analysis. PCRs were conducted in 20 µl reaction mixtures consisting of 50 ng template DNA, 1× Taq buffer, 1.5 mM $MgCl_2$, 0.4 µmol of each primer, 0.15 mM of each dNTP (dATP, dCTP, dGTP and dTTP) and 0.5 U of *Taq* DNA polymerase. Amplification was performed in an Eppendorf thermocycler (Mastercycler proS) with a program for an initial denaturation step with 94 °C for 5 min, followed by 35 cycles for 1 min at 94 °C, 1 min at 50–61 °C and 2 min at 72 °C, with a final 7-min extension at 72 °C. The PCR products were separated on non-denaturing 6 % polyacrylamide gels, stained with ethidium bromide, run under a steady 300-V voltage and then photographed under the gel documentation system (UVP GelDoc-It® Imager, Cambridge, UK). The polymorphism survey was conducted using DNA from the recipient parent PDW274 and donor parent *Ae. speltoides* pau3809. Four hundred fifty A and B genome-specific SSR markers were analyzed for the parental polymorphism survey and 210 SSR markers were found polymorphic. The selected 90 BILs were then genotyped with 210 polymorphic SSR markers.

Introgression profiling and QTL mapping

Introgression profiles of the BILs were prepared using the CSSL finder (Lorieux, http://mapdisto.free.fr/CSSLFinder/). Genotyping and phenotyping data were computed using IciMapping software for trait/QTL mapping in chromosome segmental substitution lines using single marker analysis (SMA) and stepwise regression for detecting additive QTL (Wang et al. 2012). A LOD score greater than the threshold was considered for declaring a QTL significant. The phenotypic contribution was depicted by PVE%. The phenotyping data of BILs screened under two environments, i.e., optimal and late sown, were used independently for QTL detection to detect genes/QTL expressing under heat stress.

Results

Chromosome number and pairing analysis

Meiotic analysis of F_1s from the cross of *T. durum* cv. PDW274 and *Ae. speltoides* acc. 3809 showed $2n = 21$ in all the cells (Fig. 1a). The bivalent count from 25 random cells of F_1 plants varied from two to seven with the rest of the chromosomes as univalents. Occasional trivalents were also observed in this cross combination (Fig. 1a). The chromosome number in BC_1F_1 plants varied from 26 to 28, and PMCs with 6–13 bivalents and 2–14 univalents were observed (Fig. 1b). Some of the cells had 1–2 trivalents and tetravalents also though the frequency of trivalents and quadrivalents was very less. At anaphase I, 1–4 laggards were present, which resulted in micronuclei in the tetrads generated after telophase (Fig. 1c–d).

PMC analysis of the BC_2F_1 plants showed a fairly recovered chromosome number with $2n = 27$–28 and normal chromosome pairing in most of the plants (Fig. 1e–j). Chromosome number and their configuration in 25 PMCs from each BC_2F_1 plant were analyzed and are summarized in supplementary Table S1. Frequency of the univalents and multivalents was less compared to that in BC_1F_1 plants. A well-defined equatorial plate was formed in all the PMCs, and segregation of the chromosome was normal toward the two poles except for a few laggards in some of the PMCs (Fig. 1e–j).

Temperature conditions from anthesis to maturity of wheat

The maximum and minimum temperatures in the interval between anthesis and maturity to which OE

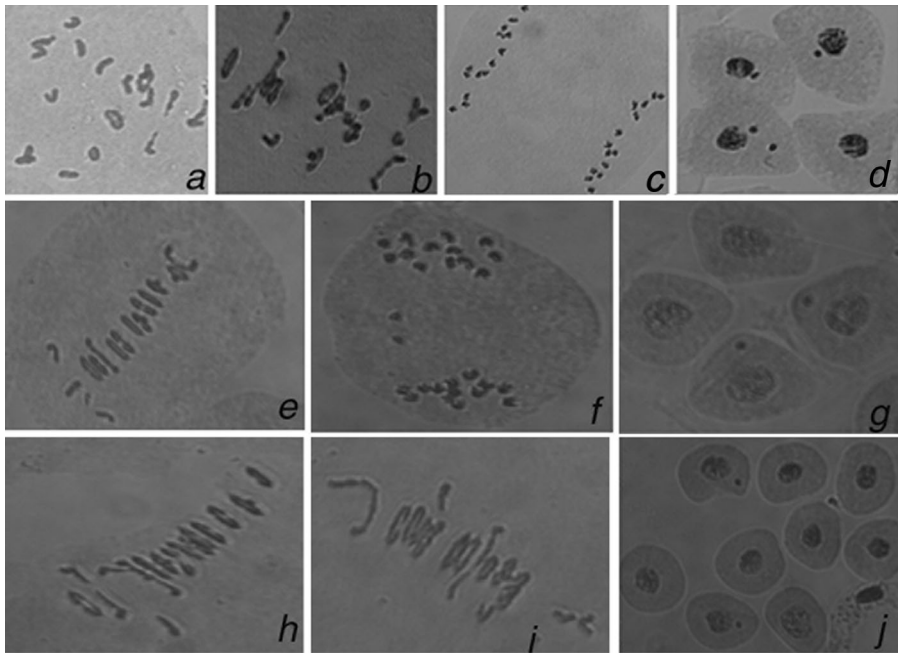

Fig. 1 Pollen mother cells of the *T. durum-Ae. speltoides* BILs at different stages of development showing chromosome number and pairing **a** F_1 of the cross *T. durum/Ae. speltoides* acc. pau3809 with $2n = 21$ ($8' + 5'' + 1'''$); **b** metaphase in BC_1F_1 plant with $2n = 28$ ($3' + 11'' + 1'''$); **c** 14:14 at anaphase in BC_1F_1 **d** a tetrad in BC_1F_1 with micronuclei varying from 0 to 2; **e**, **h** metaphase in BC_2F_1 plant with $2n = 28$ ($12'' + 4'$); **f** anaphase in BC_2F_1 plant with 13:13 + 2 laggards **i** metaphase in BC_2F_1 plant with $2n = 28$ ($3' + 11'' + 1'''$); **g**–**j** tetrads in BC_2F_1 with micronuclei only in a small number of cells

and HSE BILs were exposed recorded over the 2 years 2011 and 2012 are given in Supplementary Fig. S2. Grain filling is the most important stage for assessing the effect of heat stress on wheat yields, and for BILs grown under OE grain-filling started from the 1st week of March and continued up to the 1st week of April and for HSE BILs the 1st week of April to 4th week of April. The maximum temperature at this stage for the OE and HSE set varied from 24.8 to 33.1 and from 31.0 to 38.9, respectively, in 2011. The minimum temperatures during this period ranged across 9.8–17.4 and 14.6–24.3 for the OE and HSE set, respectively. Similar trends were recorded in 2012 as well (online Fig. S2). The difference in the temperature range in the OE and HSE set indicated that although OE BILs were also exposed to some level of heat stress, the HSE BILs experienced very high temperatures during grain filling. Even the mean vegetative period (from the date of planting to 50 % heading) in the HSE BILs was shortened by 28 and 36 days as compared to OE BILs in 2011 and 2012, respectively. Comparative analysis of the same set of genotypes under these two conditions gave a good estimation of the performance of BILs under heat stress, and the heat susceptibility index for each genotype was calculated based on the relative performance.

Phenotypic evaluation of the $BC_2F_{4\text{-}5}$ backcross introgression lines

A set of 262 BC_2F_4 backcross introgression lines (BILs) was evaluated for chlorophyll content (CCV and CCA), cell membrane thermostability (CMTV and CMTA) at the vegetative and anthesis stages and duration of the vegetative period (VP) under two environments, OE and HSE, in 2010–2011 for getting an initial assessment of the variation encompassed in the BILs. The adjusted means and range of variation in the BILs along with the parental lines is presented in online Table S2. The range of variation for chlorophyll content, cellular membrane thermostability and vegetative period in the BILs is depicted as box plots in Fig. 2a–c. CCV was significantly higher under OE

conditions as compared to HSE BILs (Fig. 2a) while the chlorophyll content of the BILs at anthesis under OE and HSE conditions was less variable than what was recorded at the vegetative stage (Fig. 2a). A number of BILs were observed as outliers in all the plots. Variation for the CMT was higher in the BILs at both the vegetative and anthesis stage, but the differences in the level of variation between OE and HSE conditions became apparent only at anthesis with OE BILs having a higher CMT than HSE BILs (Fig. 2b). The total vegetative period was significantly reduced in HSE BILs (Fig. 2c), which is expected to impact various traits important for imparting heat stress. Outlier BILs beyond the upper whisker of the box plot might be the desirable lines having *Ae. speltoides* introgressions improving the chlorophyll content and cellular membrane thermostability. Comparison of the same genotypes under OE and HSE showed significant differences in the response to heat stress imposed because of late sowing (online Table S2) for the CCV, CCA, CMTA and total vegetative period. However, no significant differences were observed for CMTV indicating that CC and CMT at anthesis can be used for screening for heat stress.

Phenotyping of the selected BILs

Based on the relative performance of different BILs for chlorophyll content and CMT under normal and heat stress conditions, a set of 90 lines was selected for evaluation in replicated trials in the subsequent season (2011–2012) for the chlorophyll content, canopy temperature, TTC cell viability and stay green habit. The data are presented in the Table 1 and Fig. 2d–f. The overall mean, minimum and maximum chlorophyll content was higher in BILs grown under heat stress, indicating that selection has led to an improvement in the chlorophyll content (Fig. 2d). Canopy temperature is an easily measured manifestation of the crop metabolic and physiological response to the environment. Eleven BILs showed a CT lower than the recurrent parent under OE conditions. Heat tolerance is also quantified by mitochondrial reduction of TTC as an acquired thermotolerance percentage. It was measured at anthesis stage, and large differences existed among tested introgression lines (Table 1), but the distribution of BILs in OE and HSE was not significantly different (Table 1; Fig. 2e); however, the range of variation was more under HSE conditions,

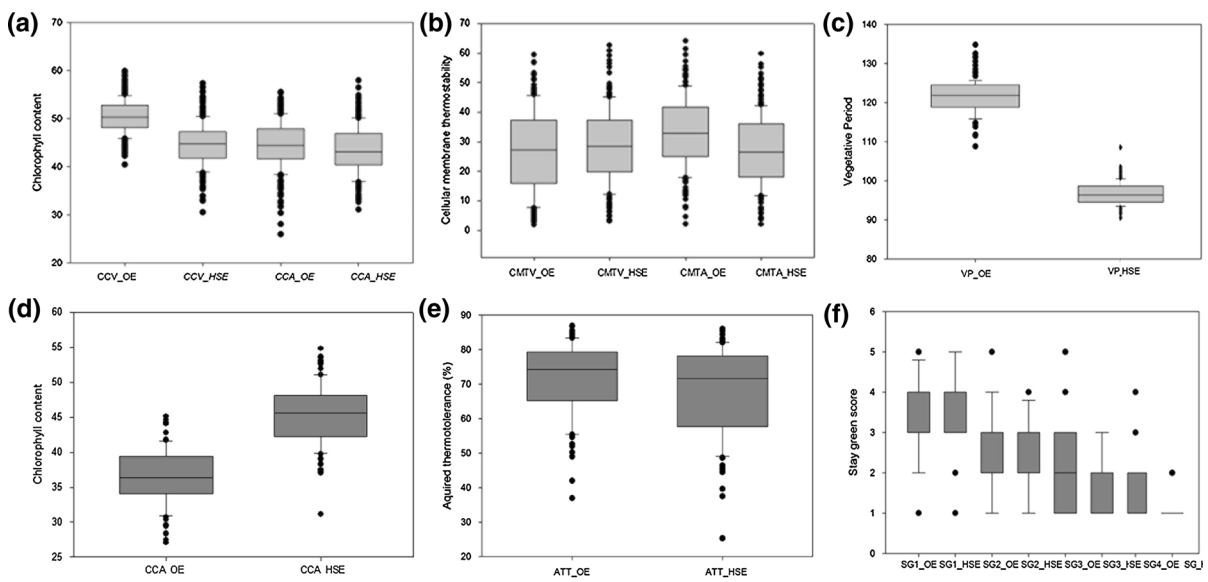

Fig. 2 Boxplots representing variation in *T. durum–Ae. speltoides* backcross introgression lines under optimum (OE) and heat stress conditions (HSE) in the random set of 262 BILs for **a** chlorophyll content at vegetative and anthesis stage; **b** cellular membrane thermostability; **c** vegetative period; selected set of 90 BILs for **d** chlorophyll content; **e** acquired thermotolerance; **f** stay green trait. *Lower*, *upper* and *middle bar of the box* represent 25, 75 % and median percentile of the variation, respectively. *Upper* and *lower whiskers* indicate *top* and *bottom* 25 % variation. *Black circles* represent the outlier BILs

Table 1 Evaluation of selected backcross introgression lines for heat tolerance components in 2011–2012 cropping seasons

Trait name	Environment	Range	Mean	SE	CV	Paired *t* value
CCA	OE	27.05–45.11	36.56	0.42	10.78	14.55 ($p < 0.001$)
CCA	HSE	31.17–54.80	45.30	0.46	9.71	
CT	OE	22.00–34.73	30.64	0.20	6.14	0 ($p = 1.000$)
CT	HSE	22.00–34.73	30.64	0.20	6.14	
ATT	OE	36.96–86.90	71.84	1.11	14.60	1.907 ($p < 0.060$)
ATT	HSE	25.33–85.99	68.04	1.37	19.16	
SG3	OE	1–5	2.04	0.10	46.92	3.145 ($p < 0.002$)
SG3	HSE	1–3	1.64	0.07	41.12	
SG4	OE	1–4	1.36	0.07	47.27	4.686 ($p < 0.001$)
SG4	HSE	1–2	1.04	0.02	19.83	

OE normal sown, *HSE* late sown

CCA chlorophyll content at anthesis, *CT* canopy temperature, *ATT* acquired thermotolerance, *SG 3–4* represent the stay-green habit recorded at the third and fourth intervals

and the number of BILs performing better than the recurrent parent was higher in the HSE set than OE. Stay green in the post-anthesis period is also an indicator of heat tolerance, which can be easily scored under field conditions for large populations. HSE BILs showed a sharp decline in stay-green duration, which might have been caused by heat stress while OE BILs had a longer stay-green duration (Fig. 2f). No significant differences existed among the OE and HSE BILs in the first two scores of the stay green, but in the third and fourth scoring BILs sown under heat stress showed accelerated senescence. HSI estimations identified 17 highly tolerant and 33 moderately tolerant BILs for chlorophyll content, 43 highly tolerant and 4 moderately tolerant for ATT and 47 highly tolerant for stay green.

Introgression profiling of the BILs

Introgression profiling of the *T. durum–Ae. speltoides* BILs using A and B genome-specific SSR markers was conducted using about 152 polymorphic markers. Introgressions were detected on both A as well as B genome chromosomes. The number of the introgressed segments varied with a minimum of six *Ae. speltoides* introgressed segments in DS132 and a maximum of 25 in DS80. The total proportion of the donor genome in different BILs varied from 6.10 % in DS132 to 27.29 % in DS29 (Supplementary Table S3). The introgression profile of the DS80 and DS29 is depicted in online Figures S3 and S4, respectively. Introgressed segments/chromosome ranged from 1 to 6 (online Table S3), and most of the BILs carried multiple introgressions on different chromosomes (Fig. 3). Out of a total 90 BILs a minimum of 73 BILs represented all the introgressions from *Ae. speltoides* onto *T. durum* chromosomes (online Fig. S5). To detect the association of the introgressed segments with heat tolerance traits, QTL mapping studies were conducted using a complete set of 90 BILs.

Mapping QTL for heat tolerance components

The mapping was conducted with single marker analysis (SMA) and a likelihood ratio test based on stepwise regression for additive QTL (RSTEP-LRT-ADD) on 90 selected BILs using phenotypic data for OE and HSE BILs individually as well as the heat susceptibility index. The results are summarized in Table 2 and Fig. 4. Three QTLs, *QCc.pau-7B*, *QCc.pau-3B* and *QCc.pau-2B*, were identified for chlorophyll content on chromosomes 7B, 3B and 2B, explaining phenotypic variation of 12.2, 13.1 and 11.7 %, respectively, with SMA as well as additive QTL mapping. Only a single QTL for canopy temperature, *QCt.pau-3B*, was mapped on 3B under both environments, which accounted for 12.8 % of the phenotypic variation. The position of *QCt.pau-3B* was almost the same as that of *QCc.pau3B*, indicating that

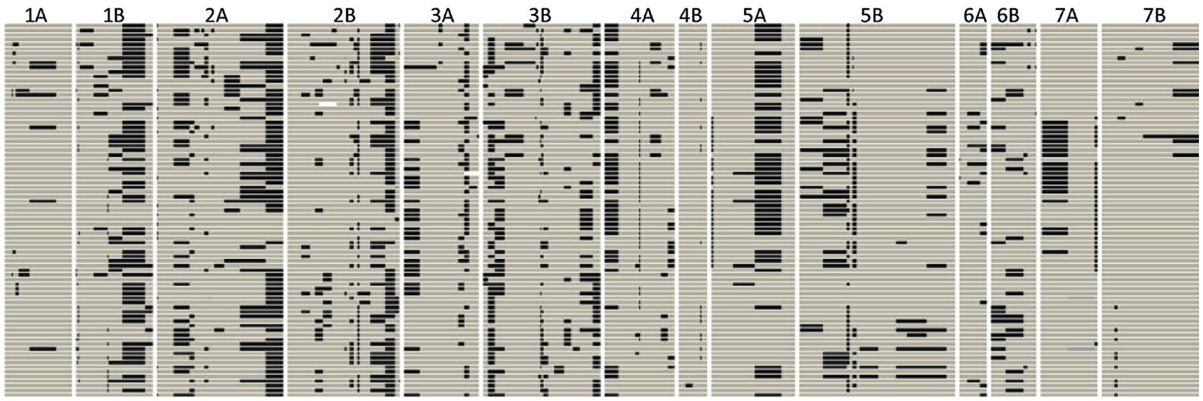

Fig. 3 The introgression profiling of the *T. durum-Ae. speltoides* BILs using SSR markers. *Gray areas* indicate *T. durum*-specific chromosomal segments, and *black areas* indicate *Ae. speltoides*-specific introgression

the same chromosomal region might be controlling both the traits. *QAtt.pau-3A* and *QAtt.pau-5B* were mapped for ATT on chromosome 3A and 5B, explaining phenotypic variation of 12.6 and 17.7 % with SMA and 14.1 and 19.2 % for additive QTL, respectively, under OE conditions. Under HSE, however, another QTL was detected on chromosome 1B with 14.2 % PVE. For HSI calculated from the relative performance of the BILS under normal and heat stress conditions, only a single QTL, *QAtt.pau-1B*, with a 16.4 % contribution, could be mapped with both SMA and additive QTL mapping. For the stay-green trait, individual data sets from the third and fourth scorings (Sg3 and Sg4) were used for QTL mapping, and different QTLs were detected for these two data sets. One QTL on chromosome 7A was mapped with SMA for Sg3 under both OE and HSE conditions as well as for the heat susceptibility index for Sg3. For Sg4, different QTLs under OE and HSE were detected with SMA on chromosome 3B and 3A, respectively. *QSg4.pau-3A* was also detected for HSI for Sg4, and it was also collocated with QTL for ATT. *QSg3.pau-7A* was also detected with the second method, which mapped additive QTL (Table 2), and this major stay-green QTL (*QSg3.pau-7A*) detected with both the methods in both the environments explained 15.9 and 14.4 % phenotypic variation, respectively, in OE and HSE. This QTL was also collocated with the vegetative period QTL *QVp.pau-7A*, which was also detected with both SMA and additive mapping. For the vegetative period, another QTL was mapped on chromosome 2B, which was collocated with a Sg3_HSI QTL (Table 2). Collocated QTL detected with SMA and additive QTL mapping are summarized in Fig. 4.

Discussion

Heat stress during grain filling duration described as terminal heat stress is one of the most important constraints affecting wheat crop productivity in most of the wheat-growing regions globally (Gupta et al. 2012). There is only limited variability within the cultivated wheat germplasm for breeding for terminal heat stress (Trethowan and Mujeeb-Kazi 2008), and wild relatives of wheat are promising as a rich resource for useful genetic variation for resistance to terminal heat stress (Ehdaie and Waines 1992; Khanna-Chopra and Viswanathan 1999; Zaharieva et al. 2001; Pradhan et al. 2012). Genetic variation for heat tolerance and associated traits and identification of donor germplasm are an essential prerequisite for breeding for heat-tolerant crops. Cultivated germplasm however would be the preferred donor option, keeping in view the ease of subsequent utilization (Gupta et al. 2010), but limited variability presents a major bottleneck. The wild germplasm, on the other hand, owing to its diverse ecogeographical distribution and adaptation to various stress prone environments, may have novel and yet untapped genetic variability. In the present investigation, we developed *T. durum-Ae. speltoides* introgression lines with enhanced heat tolerance and identified *Ae. speltoides* introgressions conferring heat tolerance.

Table 2 Summary of the QTL mapped for heat tolerance and components in *T. durum-Ae. speltoides* introgression lines using QTL mapping for CSS lines in the mapping software IciMapping

Trait	Environment	QTL	Chr.	Marker	Position (cM)	LOD	PVE (%)	Add. effect
SMA (single marker analysis)								
CCA	OE	*QCc.pau-7B*	7B	*Xwmc517*	93	2.5	12.2	−3.8
CCA	HSE	*QCc.pau-3B*	3B	*Xgwm566*	54	2.7	13.1	−4.4
CCA	OE	*QCc.pau-2B*	2B	*Xgwm148*	47	2.3	11.7	1.3
CT	OE	*QCt.pau-3B*	3B	*Xgwm264*	56	2.7	12.8	−3.2
CT	HSE	*QCt.pau-3B*	3B	*Xgwm264*	56	2.7	12.8	−3.2
ATT	OE	*QAtt.pau-3A*	3A	*Xwmc153*	87	2.6	12.6	−17.6
ATT	OE	*QAtt.pau-5B*	5B	*Xcfd60*	14	3.4	17.7	−6.2
ATT	HSE	*QAtt.pau-1B*	1B	*Xwmc269*	33	2.6	14.2	5.0
HSI_ATT	–	*QAtt_hsi.pau-1B*	1B	*Xwmc269*	33	3.8	16.4	−2.5
SG3	OE	*QSg3.pau-7A*	7A	*Xgwm471*	17	2.9	13.9	−0.4
SG3	HSE	*QSg3.pau-7A*	7A	*Xgwm471*	17	3.4	16.6	−0.3
SG3	HSE	*QSg3.pau-1B*	1B	*Xgwm269*	33	2.5	12.0	0.2
	OE	*QSg3.pau-2B*	2B	*Xgwm148*	47	2.3	13.2	0.3
HSI_SG3	–	*QSg3_hsi.pau-2B*	2B	*Xgwm148*	47	4.4	21.3	1.8
HSI_SG3	–	*QSg3_hsi.pau-7A*	7A	*Xgwm471*	17	6.5	28.7	−2.5
SG4	OE	*QSg4.pau-3B*	3B	*Xwmc787*	79	3.2	15.1	0.8
SG4	HSE	*QSg4.pau-3B*	3B	*Xwmc787*	79	2.3	11.1	0.2
SG4	HSE	*QSg4.pau-3A*	3A	*Xwmc153*	87	5.4	24.2	0.5
HSI_SG4	–	*QSg4_hsi.pau-3A*	3A	*Xwmc153*	87	4.4	20.3	−2.5
VP	OE	*QVp.pau-2B*	2B	*Xgwm148*	47	3.2	17.4	1.9
VP	OE	*QVp.pau-7A*	7A	*Xgwm471*	17	3.5	16.9	−2.3
RSTEP-LRT-ADD (likelihood ratio test based on stepwise regression for additive QTL)								
CCA	OE	*QCc.pau-7B*	7B	*Xwmc517*	93	2.5	12.2	−3.8
CCA	OE	*QCc.pau-2B*	2B	*Xgwm148*	47	2.4	11.5	1.3
CCA	HSE	*QCc.pau-3B*	3B	*Xgwm566*	54	2.7	13.1	−4.4
CT	OE	*QCt.pau-3B*	3B	Xgwm264	56	2.7	12.8	−3.2
ATT	OE	*QAtt.pau-3A*	3A	*Xwmc153*	87	3.5	14.1	−18.7
ATT	OE	*QAtt.pau-5B*	5B	*Xcfd60*	14	4.4	19.2	−6.5
ATT	HSE	*QAtt.pau-1B*	1B	*Xwmc269*	33	2.6	14.2	5.0
HSI_ATT	–	*QAtt_hsi.pau-1B*	1B	*Xwmc269*	33	3.8	16.4	−2.5
SG3	OE	*QSg3.pau-7A*	7A	*Xgwm471*	17	2.9	13.9	−0.43
SG3	HSE	*QSg3.pau-7A*	7A	*Xgwm471*	17	3.4	16.6	0.33
HSI_SG3	–	*QSg3_hsi.pau-7A*	7A	*Xgwm471*	17	6.5	28.7	−2.5
SG4	OE	*QSg4.pau-3B*	3B	*Xwmc787*	79	3.2	15.1	0.8
SG4	HSE	*QSg4.pau-3B*	3B	*Xwmc787*	79	3.3	11.6	0.2
SG4	HSE	*QSg4.pau-3A*	3A	*Xwmc153*	87	6.4	24.7	0.5
HSI_SG4	–	*QSg4_hsi.pau-3A*	3A	*Xwmc153*	87	4.4	20.3	−2.5
VP	OE	*QVp.pau-7A*	7A	*Xgwm471*	17	3.5	16.9	−2.3

CCA chlorophyll content at anthesis stage, *ATT* acquired thermotolerance, *SG* stay-green trait, *Vp* vegetative period. Threshold LODs were 2.5, 2.6, 5.3, 3.0, 2.9, 1.8, 2.6, 4.2, 7.8, 2.85 and 2.77 respectively, for CCA_OE, CCA_HSE, CT_OE, ATT_OE, ATT_HSE, SG3_OE, SG3_HSE, SG4_OE, SG4_HSE, VP_OE and VP_HSE. Map positions are according to Somers et al. (2004)

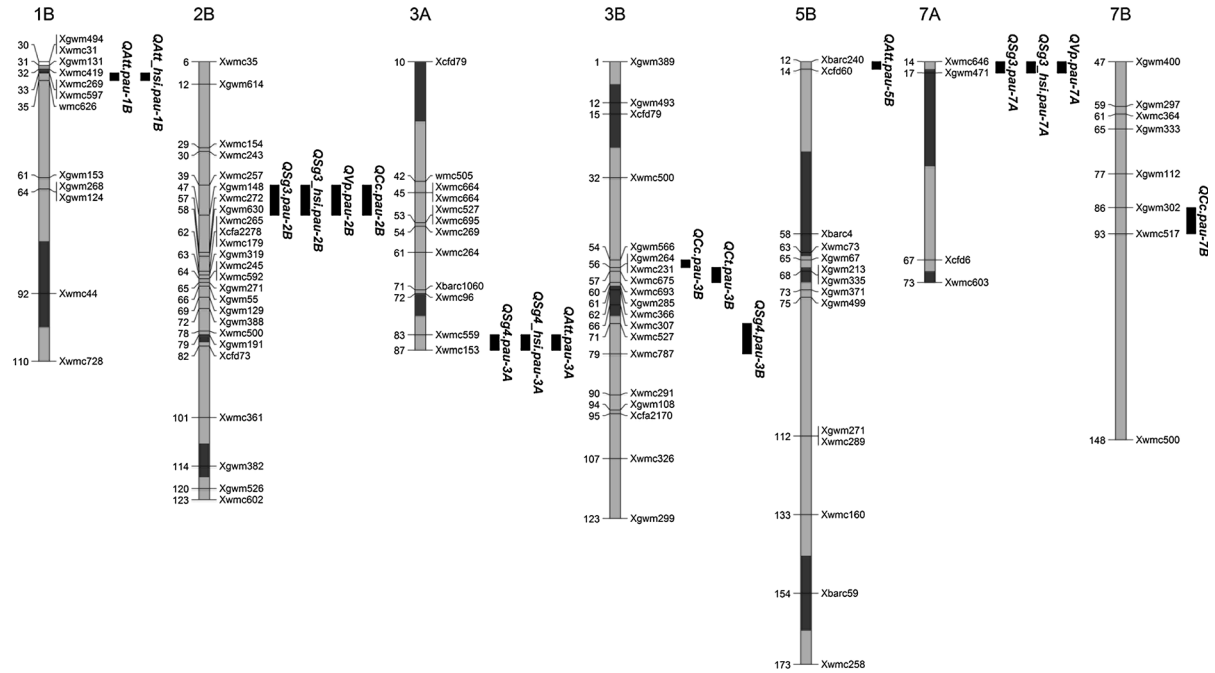

Fig. 4 Summary of the QTL detected in the *T. durum-Ae. speltoides* BILs with IciMapping software using single-marker analysis and RSTEP-LRT-ADD (likelihood ratio test based on stepwise regression for additive QTL) for chromosomal segmental substitution lines for heat tolerance-related traits. The detailed information for these QTLs is available in Table 2. The partial introgression profile map of *T. durum-Ae. speltoides* backcross introgression line DS80 has been used to depict the QTL

Development of the *T. durum-Ae. speltoides* backcross introgression lines

The S genome of *Aegilops speltoides*, the most probable B genome donor of wheat, shows very low pairing with the B genome of wheat in the presence of the *Ph1* locus in comparison to A and D genome donors *T. urartu* and *Ae. tauschii*, which show almost normal pairing with the wheat homologs (Kimber and Athwal 1972; Chapman et al. 1976; Dvorak 1976; Dobrovolskaya et al. 2011). However, high levels of homoeologous chromosome pairing have been observed in hybrids between *T. aestivum* and some accessions of *Ae. speltoides* even in the presence of the *Ph1* locus (Riley 1960; Dvorak et al. 2006), and different *Ae. speltoides* accessions have been found to have variation in the ability to elicit homoeologous chromosome pairing in hybrids with wheat (Dvorak 1972; Kimber and Athwal 1972). *Ae. speltoides* induces homoeologous chromosome pairing by suppressing the expression of the *Ph1* locus of wheat, and two major suppressors have been mapped on *Ae. speltoides* chromosomes 3S and 7S along with a minor suppressor on 5S (Dvorak et al. 2006). *Ae. speltoides* has useful variation for many traits of economic importance including tolerance for biotic and abiotic stresses. Introgression of this variation to cultivated wheat background will depend upon the induction of pairing between wheat and *Ae. speltoides* chromosomes through inhibition of the *Ph1* locus by the suppressors present in the donor accession. Very strong *Ph1* suppression activity can lead to higher level multivalents, which in turn will result in meiotic abnormalities and non-viable gametes. The chromosomes of *Ae. speltoides* accessions with a very weak *Ph1* suppressor will not pair with wheat chromosomes, and precise transfer of target traits will be difficult. *Ae. speltoides* accessions with a moderate level of suppression activity, on the other hand, will be desirable for transferring useful variability from *Ae. speltoides* to cultivated wheats. We crossed ten different accessions of *Ae. speltoides* with *T. durum*, and a viable seed set could only be obtained from two accessions (data not given), indicating that these accessions might be

carrying moderate suppression activity, which was also apparent from the partial chromosome pairing observed in triploid F_1s (Fig. 1) from crosses between *T. durum* cv. PDW274 and *Ae. speltoides* acc. pau3809. In the F_1, gametes with only a normal chromosome complement are expected to be viable, which might have led to very low seed setting observed upon backcrossing.

In F_1, since the homoeologous pairing was induced because of the presence of suppressors in the *Ae. speltoides* genome, no preferential pairing was expected with the B genome of *T. durum*, which became evident from the molecular marker analysis of BILs, which carried *Ae. speltoides* introgressions on A as well as B genome chromosomes (Fig. 3; supplementary Fig. S3 & S4). Two backcrosses with *T. durum* followed by selfing led to the normal chromosome number and pairing in the progenies. We have successfully used this strategy for transferring useful variability from other diploid progenitor species (Singh et al. 2007; Chhuneja et al. 2008; Elkot et al. 2015) in cultivated wheat where pairing between wheat and alien chromosomes was not restricted.

Evaluation of selected *T. durum-Ae. speltoides* BILs under heat stress conditions

Aegilops speltoides has been found to have a high level of thermotolerance in the limited studies conducted on this progenitor of wheat. Pradhan et al. (2012) evaluated different wild species for chlorophyll, grain number per spike, individual grain weight and grain yield under heat stress and identified *Ae. speltoides* and *Ae. geniculata* as the most heat tolerant. We evaluated different accessions of wild species of wheat for stay-green capability under temperatures as high as 39/24 °C (Chhuneja et al. unpublished) for 5 years and identified *Ae. speltoides* to be most heat tolerant in terms of the stay-green trait in the wheat gene pool. BILs developed in the present investigation depicted enhanced heat stress tolerance compared to the recipient *T. durum* cultivar assessed from the chlorophyll content, acquired thermos tolerance, canopy temperature and stay green by evaluating the same BILs under normal and heat stress conditions. Photosynthesis is sensitive to heat stress and is usually the first process that is affected by heat stress, and the chlorophyll content is the major trait linked to photosynthesis. The chlorophyll content in the selected set of BILs was much higher under heat stress conditions indicating that selection has led to the overall improvement in the chlorophyll content. HSI for chlorophyll content identified 13 BILS as highly tolerant and 33 as moderately tolerant. The HSI was also used by Yang et al. (2002) and Viswanathan and Khanna-Chopra (2001) to identify HT-tolerant wheat genotypes. A TTC-based cell viability assay was used for estimation of tolerance to heat stress. The test is based on the principle that tetrazolium salt is reduced to formazan by a respiratory dehydrogenase enzyme, which indicates the resilience of the mitochondrial component of the cells under heat stress (Chen et al. 1982; Porter et al. 1994; Fokar et al. 1998). A positive correlation between CMT and TTC tests and also with the field performance has been reported in wheat by different workers (Sadalla et al. 1990; Reynolds et al. 1994; Fokar et al. 1998; Ibrahim and Quick 2001; Dhanda and Munjal 2006). We observed highly significant differences among introgression lines for ATT and HSI estimation identified 43 BILs highly tolerant.

It is considered that genotypes with a low canopy temperature maintain high transpiration and photosynthetic rate as well as produce a high yield under heat-stressed conditions. Canopy temperature gives an indication of the interaction between the canopy of the plant and the external environment. Canopies may be cooler than the environment because they transfer relatively more heat back to the atmosphere by reflection and convection (Blum 1988). A number of studies have shown that wheat cultivars with low CT showed a trend of higher yield under heat stress (Bahar et al. 2008; Balota et al. 2008). In the present study a number of BILs performed better than the recurrent parent for CT, indicating that the selected set of BILs has better heat tolerance potential. Stay green is a vital characteristic associated with the capacity of the plant to maintain CO_2 assimilation and photosynthesis (Adu et al. 2011). It was stressed that healthy stay-green plants are more productive for grain yield (Benbella and Paulson 1998; Thomas and Smart 1993). There was a wider variation between normal sown and late sown BILs for stay green in the post anthesis period, but toward the end of the maturity the differences converged rapidly. The reduction in the stay-green trait was steep for the BILs under HSE as compared to the OE BILs, indicating high temperature exposure led to rapid senescence and HSI identified 47 lines with high tolerance to high temperature stress.

Introgression profiling and mapping heat tolerance QTL in *T. durum-Ae. speltoides* BILs

Introgression profiling of the BILs showed that *Ae. speltoides* chromosomes recombined freely with both A and B genome chromosomes of *T. durum*. The total number of introgressed segments was higher, however, in B genome chromosomes with 808 segments against 563 segments on the A genome chromosomes [homoeologous group assignments based on chromosomal locations of markers as per Somers et al. (2004)]. The reason for this difference could be that we used more SSR markers for the B genome, and hence the B genome chromosomes were more enriched. Based on introgression profiling, it was apparent that these BILs constitute a partial set of chromosomal segmental substitution lines (CSSLs). Most of the BILs carried multiple introgressions from *Ae. speltoides*. QTL mapping of the BILs indicated that chromosomes 1B, 2B, 3A, 3B, 5B, 7A and 7B are important regions associated with heat tolerance components in this set of BILs. The short arm of 3B carried the QTL for chlorophyll content as well as canopy temperature, which could be either pleiotropic effects of a single QTL or two very closely located QTLs controlling these traits. Limited studies are available on the identification of genes/QTL conferring heat tolerance or heat tolerance components such as CMS, stay green, TTC cell viability and canopy temperature (Gupta et al. 2012; Paliwal et al. 2012), but no reports on transfer and mapping of the heat tolerance QTL from wild relatives are available as per the published literature. Pinto et al. (2010) identified QTL leading to a reduction in canopy temperature on chromosome 1B, 2B, 3B, 4A and 7A in cultivated wheat. In the present study, we identified a QTL for canopy temperature on the short arm of 3B only. Wheat chromosomes 2B, 3B and 7A were identified to carry many QTLs important for heat tolerance in various earlier studies [see the review by Gupta et al. (2012)]. A higher duration of the "stay-green" habit has been reported to have a positive effect on grain yield under high temperature in both bread and durum wheats (Benbella and Paulsen 1998; Hafsi et al. 2000; Rahman et al. 2005; Foulkes et al. 2007; Vijayalakshmi et al. 2010). Three QTLs for stay green, one each on the chromosome arms 1AS, 3BS and 7DS, were reported by Kumar et al. (2010). In *T. durum-Ae. speltoides* BILs we however detected the stay-green QTL on chromosome 2B, 3A, 3B and 7A, which were expressed under both optimal and heat stress conditions. For the total vegetative period of the BILs QTLs were detected on chromosome 2B and 7A, which were collocated with the QTL for stay green on these chromosomes. Positive alleles of the target QTL contributed by both donor as well as recipient parents were detected. Chlorophyll and stay-green QTLs on chromosome 2B, acquired thermotolerance QTL on 1B and stay-green QTL on chromosome 3B were contributed by *Ae. speltoides*. Overall, in this set of 90 BILs we detected a total of 15 QTLs for various physiological traits contributing to heat tolerance under optimal and/or heat stress conditions using two different mapping strategies.

In addition to these physiological traits, the *T. durum-Ae. speltoides* BILs also carry variation for many other useful traits such as resistance to leaf rust and stripe rust, foliar blights, spike size, grain size and some developmental traits (data not given). The BILs as well as QTL identified in the present investigation form a germplasm base and a genomic resource for transfer of heat stress tolerance to elite wheat germplasm. Development of the near isogenic lines for single introgressed segments is in progress.

Conclusion

Heat stress tolerance has been transferred from *Ae. speltoides* to *T. durum*, exploiting the *Ph1* suppressors of *Ae. speltoides*, which led to the pairing of the S genome with the A and B genome of *T. durum*. *Ae. speltoides* introgressed regions carrying genes/QTL for physiological traits conditioning the heat stress response have been identified. The BILs identified to be heat stress tolerant can be used as donor stocks for improving the heat stress tolerance of hexaploid wheat using the markers linked to the target QTL. As per published evidence, this is the first report of the utilization of *Ae. speltoides* as a donor parent for improving the heat tolerance in cultivated wheat.

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Compliance with ethical standards

Competing interest The authors declare that they have no conflict of interest.

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